IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: William D Huse *et al.*
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Filed: 11/04/99 Examiner: L. Helms
Entitled: **Methods of Optimizing Antibody Variable Region Binding Affinity**

DECLARATION OF DR. JEFFRY D. WATKINS PURSUANT TO 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING UNDER 37 C.F.R. § 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Dated: March 22, 2002 By: Traci E. Light

I, Dr. Jeffry D. Watkins, under penalty of perjury state that:

1. I am a joint inventor of the subject matter claimed in the above-captioned United States Patent Application. I am presently employed as the Vice President of Research at Applied Molecular Evolution, Inc. (formerly Ixsys, Inc.), the Assignee of the above-captioned United States Patent Application.

2. I understand that in the context of the present invention, the Examiner has raised issues as to i) whether the oligonucleotides encode only framework regions (FRs) or only complementarity-determining regions (CDRs), and whether the oligonucleotides hybridize in any order, ii) whether the structure of an individual chain of an antibody variable region, as claimed in the pending claims, requires three CDRs in proper order with the intervening FRs and iii) the precise meaning of a "portion" of the molecules as claimed in the pending claims. The following paragraphs address each of these concerns, in order.

3. Overlapping oligonucleotides hybridize due to complementary sequences, which are present at the ends of adjacent oligonucleotides. Thus, hybridization of overlapping

oligonucleotides occurs in an orderly manner along the length of the assembled heavy and light chains, as dictated by the presence of appropriate complementary sequences at the ends of each oligonucleotide. It is the presence of these sequences which permit the "overlap" to occur between oligonucleotides encoding adjacent regions of the heavy and light chains. The oligo design is dependent on the particular construct. "FR" oligos may encode flanking CDR sequences at one or both ends, and "CDR" oligos may encode flanking FR sequences at one or both ends.

One example of overlapping oligonucleotides is Rosok *et al.* ["A Combinatorial Library Strategy for the Rapid Humanization of Anticarcinoma BR96 Fab" *J. Biol. Chem.* 271:22611 (1996), which was provided in the Information Disclosure Statement submitted on June 2, 2000, and an additional copy of which is provided at Tab 1].

4. With respect to antibody variable region structure, the heavy and light chains of an antibody variable region each include four FRs and three CDRs, which are in a defined order : FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. The specific example described in the specification as filed (page 9, line 22- page 10, line 2) demonstrates this.

5. With respect to "portions" of molecules, in the field of molecular biology, a portion of a molecule refers to a segment, section, part or a fragment of a molecule or sequence. The specification demonstrates the use of the term "portion" to refer to a fragment of a molecule or sequence (*i.e.* to refer to some part of the molecule or sequence, and is not the entire molecule or sequence). For example, on page 10, in the definition of "donor" (lines 10-23), it is clear that a "portion" can be derived from a parent antibody molecule, or fragment thereof. Similarly, on page 10, lines 24-27, in the definition of "acceptor", it is clear that the acceptor is to receive a donated "portion" of a parent or donor antibody (or fragment of a parent or donor antibody molecule). Also, on page 11, lines 4-10, it is clear that an antibody variable region can be an intact amino terminal "portion" of an antibody, or functional fragments thereof. Finally, on page 23, lines 21-25, reference is made to a "significant portion" of a CDR maintaining the authentic donor CDR sequence.

6. The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Dated: 03-22-02

Jeffry Watkins, Ph.D.

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A Combinatorial Library Strategy for the Rapid Humanization of Anticarcinoma BR96 Fab*

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Mae Joanne Rosok‡§, Dale E. Yelton‡, Linda J. Harris‡, Jürgen Bajorath‡, Karl-Erik Hellström‡, Ingegerd Hellström‡, Gina A. Cruz¶‖, Karin Kristensson¶, Huey Lin¶, William D. Huse¶, and Scott M. Glaser¶**

From ‡Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121 and ¶Ixsys, Inc., San Diego, California 92121

We have used a combinatorial mutagenesis strategy to humanize BR96, a monoclonal antibody that binds to the Lewis Y class of tumor antigens. This approach allows simultaneous assessment of hundreds of humanized variable regions to identify the molecules that best preserve affinity, thus overcoming the major drawback of current humanization procedures, the requirement to construct and analyze each humanized antibody separately. Murine residues of BR96 were mutated to human if they were solvent-exposed residues that did not participate in the formation of the antigen binding site and were not at the interface of the light and heavy chain. At positions that might be involved in binding to antigen, the choice between the murine and human residue was more difficult. Murine and human alternatives were incorporated into a combinatorial library at positions representing buried residues that might affect the structural integrity of the antigen binding site. By encoding this library of humanized BR96 Fabs in an M13 phage vector, we rapidly identified several candidates with nearly identical antigen binding, within 2-fold, of the chimeric Fab. Additional mutagenesis directed at sites suggested in the literature as potentially important for antigen binding in a similar anti-Lewis Y antibody yielded no further improvements.

The human immune response to antigenic sequences in rodent monoclonal antibodies (mAbs)[1] has limited the therapeutic use of these proteins (1, 2). The creation of chimeric antibodies with the foreign variable regions (V regions) joined to human constant regions (3–5) has addressed this limitation, in part. Many chimeric antibodies, however, continue to induce an immune response directed to the foreign V regions (6, 7), and the immunogenicity of a given V region in humans is not predictable at present. The desire to further minimize the potential for immunogenicity of any xenogeneic V region has motivated the development of several methods for "humanizing" an antibody. "Humanization" involves changing foreign framework residues rare in human V region sequences to residues more commonly found. The involvement of complementarity determining regions (CDRs) in antigen binding generally requires preservation of these sequences.

Winter and colleagues (8–10) first described a method for transferring the specificity of a murine antibody by grafting its CDRs onto human framework regions. The simple grafting of CDRs onto human frameworks, however, often results in significantly reduced affinity for antigen (9, 11). Although humanization is straightforward in principle, in practice alterations at framework residues, particularly those interacting with CDRs, frequently affect antigen binding. Analysis of antibody-antigen complexes by x-ray crystallography has shown that some framework residues can interact directly with antigen (12), affect the conformation of the CDR loops (11, 13, 14), and influence packing interactions between the β-sheet strands (15). Molecular modeling and crystal data, when available, have helped to identify murine framework residues that most likely contribute to the integrity of the binding site. Preservation of these residues in humanized antibodies often maintains affinity for antigen comparable to the original antibody (9, 11, 14, 16). Detailed structural information is not available for many antibodies, which prompted development of other approaches for designing humanized V regions that maintain acceptable affinity. Such methods include computer assisted design (17, 18), variable domain resurfacing (19), framework exchange (20), a positional consensus method (21), and sequence homology comparisons (22).

These humanization methods generally require an iterative approach to create, characterize, and correct, if necessary, the sequence of a humanized V region. Even as our understanding of antibody structure increases and more information is available regarding positions that predominantly affect ligand binding, it remains difficult to generalize the approach. Designing a humanized antibody requires making choices of which residues to change and which to retain. Making these decisions is often subjective, but creating a set of humanized molecules that represents all of the alternatives at positions where the choice is difficult reduces the degree of subjectivity. Simultaneously determining the binding activity of the many different humanized forms contained in such a set or "library" reduces or eliminates the need to rely on a trial and error strategy.

We have humanized the anticarcinoma mAb BR96 by a combinatorial library design strategy that created a set of humanized V regions from which sequences that best preserved affinity were rapidly selected. BR96 recognizes a tumor-associated antigen expressing a Lewis Y (Le^y) related carbohydrate on the

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§ To whom correspondence and reprint requests should be addressed: Bristol-Myers Squibb Company, 3005 First Ave., Seattle, WA 98121. Tel.: 206-727-3551; Fax: 206-727-3603.

‖ Current address: Desmos, Inc., 3550 General Atomics Court, San Diego, CA 92121.

** Current address: Bristol-Myers Squibb, 3005 First Ave., Seattle, WA 98121.

[1] The abbreviations used are: mAb, monoclonal antibody; V region, variable region; CDR, complementarity determining region; Le^y, Lewis Y; PCR, polymerase chain reaction; sLe^y, synthetic Le^y tetrasaccharide hydrazide; HRP, horseradish peroxidase; SPR, surface plasmon resonance; k_{off}, dissociation rate constant; sFv, single chain variable fragment; ELISA, enzyme-linked immunosorbent assay.

surface of many human carcinomas (23, 24). An immunoconjugate of chimeric BR96 with doxorubicin induces complete regressions and cures of human tumor xenografts growing in athymic mice (25). The chimeric BR96 immunoconjugate is currently in human clinical trials. We have constructed an M13 phage Fab expression library containing combinations of human and murine residues at several positions among those selected for humanization of BR96. In this report we discuss the design of a humanized BR96 library and the selection and characterization of several humanized BR96 Fabs from it. In addition, we compare our humanization procedure and results with those of Benhar *et al.* (20), who humanized B3, another anti-Le^y mAb (26) that has a highly homologous sequence to BR96 (27).

EXPERIMENTAL PROCEDURES

Selection of Human V Region Framework Sequences—Human immunoglobulin sequences were identified from several protein data bases, GenBank™, EMBL, SwissProt, and PIR, by the Stringsearch program of the GCG Package (Sequence Analysis Software Package, Genetics Computer Group, Inc., Madison, WI). To compare the human sequences to BR96 V regions we used the FastA program (28) adapted for the GCG package. We selected the human germline light and heavy chain sequences with the highest degree of homology with the BR96 framework regions 1, 2, and 3 of each chain by evaluating variable domain sequence profiles as described previously (29).

Combinatorial Humanized BR96 Antibody Library Design—BR96 variable domain heavy and light chain gene sequences and the corresponding selected human sequences were aligned, and CDR loop assignments were made according to the canonical structure model described by Chothia and Lesk (30). The structure-based CDR loop assignments of Chothia and Lesk (30) spatially delineate the binding site better than the sequence-defined hypervariable regions of Kabat *et al.* (31). Sequence numbering and framework region alignments were according to Kabat *et al.* (31). Differences in residues between BR96 and selected human germline templates were mapped on a three-dimensional model of the BR96 variable regions (32). From the model we identified residues important (13) or potentially important to the interface of the heavy chain and light chain V regions or to the integrity of the antigen binding site. The selected human framework sequences were then inspected for conservation of buried ("packing") residues as defined by Padlan (33). Considering the modeling information and identification of buried residues, we made decisions to retain a murine residue, change it to the human counterpart, or include both the human and murine alternatives in the combinatorial library.

Oligonucleotide Synthesis—Oligonucleotides were synthesized and purified as described elsewhere (34). Synthesis of oligonucleotides incorporated a 1:1 mixture of murine codons to human codons at the selected library positions (35). The oligonucleotide mixtures were double-deprotected with concentrated NH_4OH, purified by polyacrylamide gel electrophoresis, and eluted from the excised gel slices (36).

Library Construction—The molecule chosen for humanization was a mutant of chimeric BR96, M1, that contains an Asp to Ala mutation at heavy chain position 97 resulting in an increased affinity for tumor antigen (34). We generated *de novo* full-length humanized V region genes from long overlapping oligonucleotides for insertion into an M13 Fab expression vector by hybridization mutagenesis (35, 37). We included codons for the murine and human alternatives at library positions in the synthesis of the humanized V region oligonucleotides, rather than create the combinatorial library after the V regions were incorporated into the phage vector (34). The full-length humanized V region single-stranded DNAs required for hybridization mutagenesis were synthesized in a two-step polymerase chain reaction (PCR). Annealing PCR primers had at least 18 nucleotide residues complementary to vector sequences for efficient annealing of the single-stranded V region products to the vector. Equimolar concentrations of the annealing PCR primers and of six (light chain) or seven (heavy chain) overlapping 60–80 nucleotide sequences were amplified to generate double-stranded DNA. From the double-stranded products single-stranded DNA was prepared by asymmetric PCR. The oligonucleotides for the light and heavy chains with the sites of library positions *underlined* are as follows. Light chain oligonucleotides were forward primer (5′), 5′-GCCCAACCAGCCATGGCCGATGTTGTCATGACCCAA-3′; reverse primer, (3′), 5′-GATGAAGACAGATGGTGCAGCCACAGTACGTTTGAT-3′. Oligonucleotide 1 (forward): 5′-GATGTTGTCATGACCCAAACCCCACTGTCC (AGT/CTT)CCTGTCACGCTTGGACAACCTGCGTCCATCTCTTGC-3′; 2 (reverse): 5′-TTCCAGATAGGTGTTGCCATTATTATGTACAATGATCTGACTAGATCTGCAAGAGATGGACGC-3′; 3 (forward): 5′GGCAACACCTATCTGGAATGG(CTG/TAC)(CAG/CTG)CAGAGACCAGGCCAG (CCT/TCT) CCACGGCTCCTGATCTACAAAGTTTCC-3′; 4 (reverse): 5′-TCTCCCAGCTCCACTGCCGCTGAACCTGTCTGGGACCCCAGAAAATCGGTTGGAAACTTTGTAGATCAGG-3′; 5 (forward): 5′-AGTGGAGCTGGGACAGATTTCACACTCAAGATCAGCAGAGTGGAGGCTGAGGATGTGGGAGTTTACTACTGCTTCCAG-3′; 6 (reverse): 5′-AGCCACAGTACGTTTGATTTCCA(C/A)CTTTGTCCCTTGGCCGAACGTGAATGGAACATGTGAACCCTGGAAGCAGTAGTAAAC-3′. Heavy chain oligonucleotides were forward primer (5′), 5′-ACCCCTGTGGCAAAAGCCGAAGTGCAACTGGTGGAG-3′; reverse primer (3′), 5′-TGGGCCCTTGGTGGAGGCGGAAGAGACCGTGACCAG-3′; oligonucleotide 1 (forward): 5′-GAAGTGCAACTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCCTGCGACTTTCCTGT-3′; 2 (reverse), 5′-CTGGCGAACCCAATACATGTAATAGTCACTGAAAGTGAATCCAGATG(T/C)AGCACAGGAAAGTCGCAG-3′; 3 (forward), 5′-TATTGGGTTCGCCAGGCTGGAGGCAAGGGACTGGAGTGGGTC(G/T)CATACATTAGTCAAGGTGGTGAT-3′; 4 (reverse), 5′-GTCTCTGGAGATGGTGAATCGACCCTTTACGGAGTCTGCATAGTCGGTTATATCACCACCTTGACT-3′; 5 (forward), 5′-ACCATCTCCAGAGACAATGCAAAGAACAGCCTGTACCTGCAAATGAACAGCCTGAGGGACGAGGAC-3′; 6 (reverse), 5′-AGCAAACCAGGCCCCGTCCGCCAGGCCTGTTGCACAGTAATACACGGCTGTGTCCTCGTCCCTCAG-3′; 7 (forward), 5′-GGGGCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACGGTCTCTTCC-3′.

The PCR reaction (50 μl) for each chain contained 50 pmol each of oligonucleotides 1–6 (light chain) or oligonucleotides 1–7 (heavy chain), and the respective forward (5′) and reverse (3′) annealing PCR primers, 67 mM Tris-HCl, pH 9.2, 1.66 mM NH_4SO_4, 2 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM dNTPs and 0.25 unit *Taq* DNA polymerase (Boehringer Mannheim). Conditions for PCR amplification were denaturation for 30 s at 94 °C, annealing for 30 s at 50 °C, and synthesis at 72 °C for 30 s for 2 cycles, followed by 30 cycles of denaturing for 30 s at 94 °C, annealing for 30 s at 55 °C, and synthesis for 30 s at 72 °C and extension for 5 min at 72 °C. A 2-μl portion (approximately 0.8 μg) of the double-stranded DNA products was subsequently amplified by asymmetric PCR (100 μl) (35, 38) adding only the reverse annealing PCR primer (0.1 nmol) in the same buffer described above without β-mercaptoethanol. The reaction was denatured at 95 °C for 1 min followed by 20 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s, and synthesis at 72 °C for 30 s, followed by extension for 7 min at 72 °C. The single-stranded products were isolated in a 2% low melting temperature agarose gel prepared with Tris acetate EDTA buffer and then extracted from the gel with the Geneclean kit according to the manufacturer (San Diego, CA). 150 ng of the light chain and heavy chain single-stranded PCR products were phosphorylated with polynucleotide kinase and simultaneously annealed to 250 ng of uridinylated M13IX104 vector (37, 39). M13IX104 encodes C_H1 of human IgG_1 and human κ light chain constant domain. The reaction mixture was extended and ligated as described (40) and diluted with water to a final volume of 20 μl.

Screening of Immunoexpression Library and Binding Analysis of Humanized Fabs—Escherichia coli strain XL-1 was transfected by electroporation with 1 μl of the mutagenesis reaction and incubated at 37 °C until plaques had formed. Plaque lift assays were performed as described using 2 μg/ml synthetic Le^y tetrasaccharide hydrazide (sLe^y) (Alberta Research Council (ARC), Edmonton, Alberta, Canada) conjugated to horseradish peroxidase (sLe^y-HRP) as a probe (34). Phage clones reactive with sLe^y were used to infect cultures of *E. coli* strain MK30-3 (Boehringer Mannheim) for the production of soluble Fab fragments. To characterize each humanized Fab we prepared periplasmic fractions, performed quantitative and antigen binding ELISAs with paraformaldehyde fixed tumor cells or tumor cell membranes, and conducted surface plasmon resonance (SPR) experiments as described previously (34). As a control for ELISA and SPR experiments, BR96 Fab was prepared by proteolytic digestion of chimeric BR96 with papain using the ImmunoPure® Fab Preparation Kit (Pierce) according to the manufacturer's instructions.

DNA Sequence Analysis—Single-stranded DNA from humanized BR96 clones was isolated, and the heavy and light chain V region genes were sequenced by the dideoxynucleotide termination method (Sequenase Version 2, U. S. Biochemical Corp., Cleveland, OH).

Site-directed Mutagenesis—Site-directed mutagenesis to correct PCR errors or to introduce additional specific single codon changes was performed essentially as described by Kunkel *et al.* (40). The template

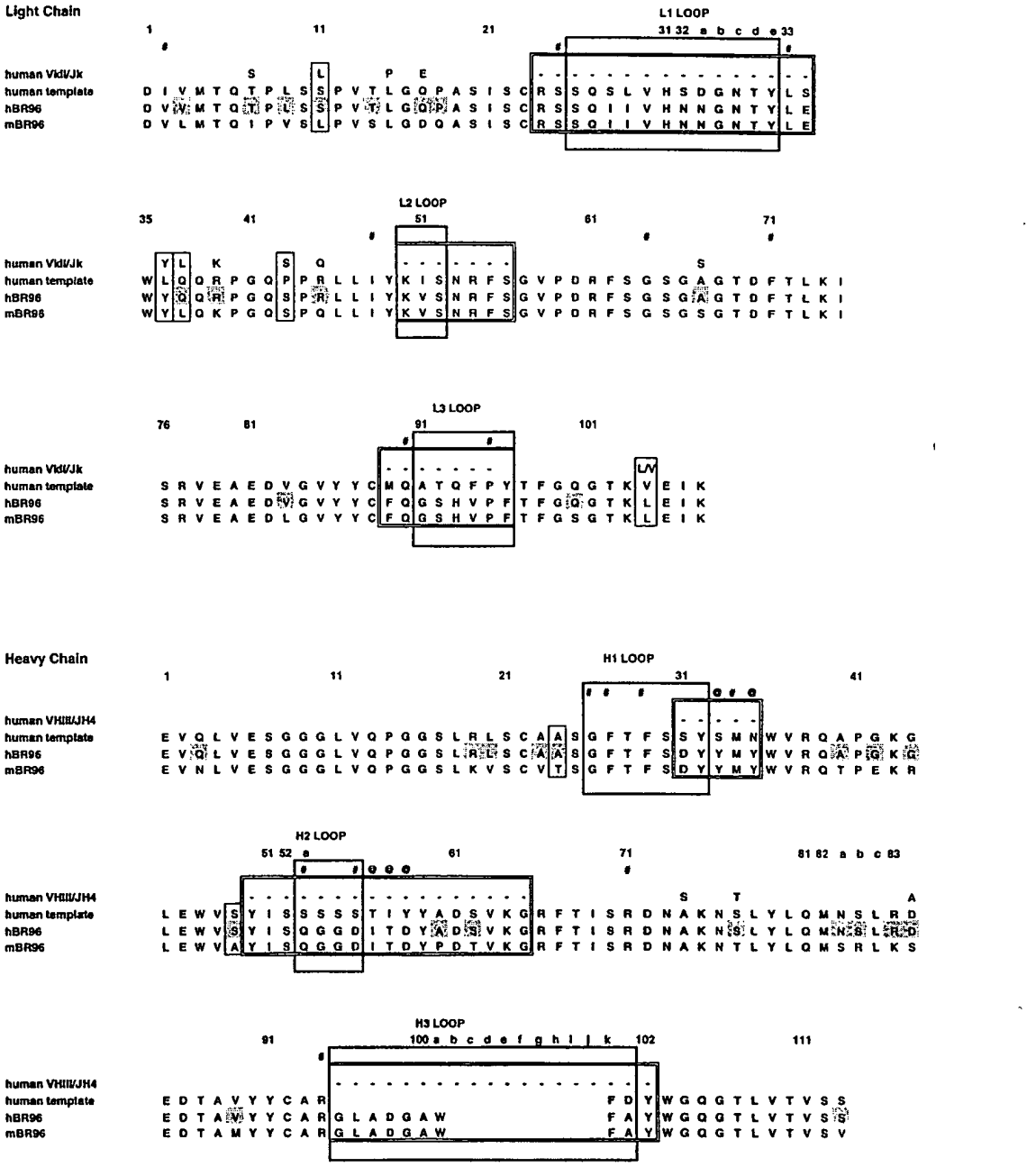

FIG. 1. **Alignment of murine BR96 M1, humanized BR96, and human template sequences.** *A*, alignment of murine BR96 M1 (*mBR96*), humanized BR96 M1 (*hBR96*) light chains, and the human light chain template; *B*, alignment of mBR96, hBR96 heavy chains, and the human heavy chain template. The accession number for each of the human templates is provided in the text. The humanized BR96 sequence, hBR96, is from clone 2-40. *Solid line boxes* enclose the CDR loops of the light and heavy chains. The designations for light chain CDRs are *L1*, *L2*, and *L3*, and for the heavy chain are *H1*, *H2*, and *H3*. A *double line* outlines the hypervariable regions as defined by Kabat *et al.* (31). The *shaded areas* indicate humanized residues and a *single line* encloses the library positions. # designates structural determinant residues (13) and @ refers to murine residues maintained because they were juxtaposed to a CDR loop.

for site-directed experiments was uridinylated single-stranded DNA from humanized Fab clone (2-40) with two affinity mutations. This affinity mutant, designated M3, had mutations in CDR2 ($Gly^{53} \rightarrow Asp^{53}$) and CDR3 ($Asp^{97} \rightarrow Ala^{97}$) of the heavy chain, which improved the binding affinity of chimeric BR96 for tumor antigen (34). Two site-specific mutants of humanized BR96 M3 were prepared by introducing mutations at position 19 or 82b in the heavy chain. The oligonucleotide sequences to mutate Arg to Lys at position 19 and Ser to Arg at position 82b in the heavy chain of humanized BR96 M3 were 5′-GATGCAGCACAGGATAACTTCAGGGACCCTCCAG-3′ and 5′-CGTCCCTCAGTCGGTTCATTTG-3′, respectively.

RESULTS

Construction of a Combinatorial Humanized BR96 Fab Antibody Library—To design the humanized BR96 framework V region we selected a set of human germline sequences from a homology search of available immunoglobulin V region sequences. Because framework residues can influence the conformation of the antigen binding site, we chose human frameworks closely resembling that of murine BR96 (mBR96) to maximize the probability they would provide similar structural

TABLE I
Construction of combinatorial humanized BR96 M1 Fab library
The murine and human amino acids at buried residue positions included in the humanized BR96 M1 combinatorial library are listed.

	Mouse	Human
Heavy chain positions		
24	Thr	Ala
49	Ala	Ser
Light chain positions		
11	Leu	Ser
36	Tyr	Leu
37	Leu	Gln
43	Ser	Pro
104	Leu	Val/Leu

support of the CDR loops. We preferred germline sequences for templates rather than same or higher scoring non-germline sequences, because of the unknown contribution of somatic mutations to immunogenicity. Since most light and heavy chain combinations assemble correctly, we chose the most homologous templates for each chain independently. The chosen human heavy chain V region, locus HSIGDP51 (accession no. Z12351), subgroup III, had 80% homology of frameworks 1, 2, and 3 to the BR96 heavy chain sequence (Fig. 1). The selection for the light chain template was germline V region HSIGVA23 (accession no. X12684), subgroup II, with 79% homology to the BR96 light chain frameworks 1, 2, and 3 (Fig. 1). The most homologous J region segments were JH4 for the heavy chain and Jk2 for the light chain.

The antibody humanized in these studies was BR96 M1, a mutant of BR96 with improved affinity resulting from one amino acid substitution in CDR3 of the heavy chain (34). The heavy and light chain amino acid sequences of BR96 M1 and human template sequences were aligned and differences between the murine and human frameworks identified. We inspected a BR96 model (32) to map the predicted spatial position of each residue, and then decided whether to retain the murine residue, to substitute the human residue, or to include both the murine and human residues in the combinatorial library. We maintained the murine residues at structural determinant positions (marked with # in Fig. 1) that are responsible for the canonical loop conformations of an antibody (13). The only structural determinant position in the framework with a different residue in BR96 and the human template was position 2 in the light chain (Fig. 1), and we retained the murine residue valine. Examination of the model indicated that none of the solvent exposed residues different between BR96 M1 and the human template were likely to be important for maintaining the structure of the antigen binding site. We chose to mutate all solvent exposed residues, 11 residues in the light chain and 16 in the heavy chain, to the human amino acids (Fig. 1).

The residues considered for library positions were buried amino acids as defined by Padlan (33). Humanization of the anti-lysozyme antibody, D1.3 (11) and of an anti-CD18 mAb, m1B4 (22) demonstrated the critical nature of some buried residues. CDR grafting of D1.3 caused a 45-fold decrease in its binding affinity. Changing four buried framework residues back to the murine residues restored binding activity to within 4-fold of the murine antibody. For humanized 1B4, recovery of a 3-fold loss of activity measured in an *in vitro* adhesion assay occurred by changing three buried residues back to the murine residues (22).

The BR96 library included buried residues at heavy chain and light chain positions where the murine and human choices were between nonconservative residues. In the heavy chain of BR96 four buried positions differed between the murine (m) and human (h) template sequences, position 20 (m = Val, h = Leu), 24 (m = Thr, h = Ala), 49 (m = Ala, h = Ser), and 77 (m = Thr, h = Ser). We included positions 24 and 49 (Table I) in the library, since they are less conservative choices. The amino acid choices at positions 20 and 77 were conservative and we chose to humanize both. In addition, at position 20, the murine residue Val rarely occurs in human VHIII sequences (less than 5%) (31). The fourth potential library position, 77, is not proximal to a CDR loop and the computer model suggested that neither the Thr (murine) nor Ser (human) would affect CDR conformation.

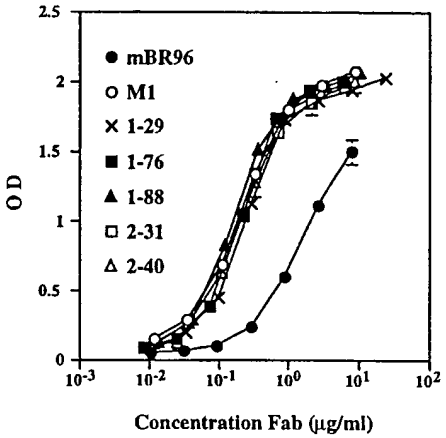

FIG. 2. **Binding of humanized BR96 Fabs to H3396 tumor cell membranes.** Periplasmic fractions containing Fab were prepared from *E. coli* cultures infected with phage clones chosen from the humanized BR96 Fab combinatorial library or infected with parent BR96 M1 phage. The clones are designated by number. The concentration of Fab in a periplasmic preparation was measured in a quantitative ELISA (34). mBR96 Fab was proteolytically derived by papain digestion from whole chimeric BR96 immunoglobulin. For this figure and Fig. 4 the standard deviation of the duplicates at each data point is indicated by *error bars*, which are often smaller than the data point symbol.

Inspection of buried residues in the light chain V regions revealed different residues at six positions, position 2 (m = Thr, h = Ser), 11 (m = Leu, h = Ser), 36 (m = Tyr, h = Leu), 37 (m = Leu, h = Gln), 43 (m = Ser, h = Pro), and 104 (m = Leu, h = Val). As noted above we maintained the murine residue at position 2 of BR96 because this position is a canonical structural determinant for the L1 loop (13). Even though the two amino acid choices are conservative at position 104, we included this position in the combinatorial library since representation of Leu and Val in the human Jk2 segment is approximately equivalent in human J region sequences homologous to BR96. Therefore, the humanized antibody library incorporated the murine and human alternative amino acids at light chain positions 11, 36, 37, 43, and 104 and at heavy chain positions 24 and 49 (Table I).

We constructed the combinatorial library of humanized BR96 M1 in an M13 Fab expression vector. *De novo* synthesis of the humanized V region genes by PCR amplification of long overlapping oligonucleotides was used so that all combinations of the alternatives at library positions were introduced in a single reaction. The single-stranded DNAs, produced by a second, asymmetric PCR, were incorporated into the uridinylated M13IX104 phage vector by hybridization mutagenesis (37, 39). Inclusion of codons for the murine and human alternatives at library positions into the oligonucleotides yielded PCR products representing all permutations. The resulting library contained 2^2 different heavy chains and 2^5 different light chains for a total of 128 variant humanized BR96 M1 Fab molecules. If desired, substantially larger libraries can be created by this method to examine alternate residues at many more positions.

Selection and Characterization of Humanized BR96 Fabs—Screening of the combinatorial library by a nitrocellulose filter

lift assay identified phage expressing Fab that bound to the sLe^y antigen, a synthetic tetrasaccharide we believe to represent the minimal elements of the Le^y antigen expressed on tumor cells (41). This primary screen eliminates phage that do not express or poorly express Fab fragments due to mutations introduced into the library or by PCR error. We found that overlapping oligonucleotide gene synthesis introduced deletion mutations, which we also eliminated by this primary screen for function. The complexity of this library is relatively small (128 members) compared to typical combinatorial libraries constructed for affinity maturation or discovery of new specificities (10^8–10^{11} members) (34, 42). Therefore, screening the library completely is technically simpler. Even so, to ensure examination of each of the mutant combinations among the background of nonproductive constructs we screened several hundred phage plaques. We selected 150 clones with strong staining in an sLe^y-HRP plaque lift to characterize by ELISA on fixed H3396 tumor cells. Of those clones we chose 15 with binding similar to the murine V region Fab parent to quantitatively assess binding to antigen. The concentration of Fab fragments in periplasmic fractions of infected *E. coli* cultures was measured by ELISA, and the relative binding activities of each Fab was assessed on H3396 tumor cell membranes. All the Fabs from the selected clones bound to tumor antigen comparably to the parental murine BR96 M1 Fab. Binding of five representative humanized clones to H3396 tumor membranes is shown in Fig. 2. The high affinity chimeric BR96 mutant M1 has approximately 8–10-fold improved binding to tumor antigen compared to the chimeric BR96 Fab (34) (Fig. 2). All five clones selected from the humanized combinatorial library preserved this improvement as assessed by ELISA.

These five humanized BR96 M1 Fabs were analyzed by SPR to compare their dissociation rates from sLe^y (Table II). Plasmon resonance measures the kinetic parameters of antibody-antigen binding and yields a more sensitive measurement of binding activity than the ELISA. Previous SPR studies with purified BR96 Fab and its high affinity mutants showed that the difference in binding affinity constants among the mutants was primarily a function of the dissociation rate constant (k_{off}) (34). The k_{off} for the humanized clones ranged from 0.069 s^{-1} to 0.064 s^{-1}, and the k_{off} of the parental BR96 M1 was 0.041 s^{-1}. Because the ELISA data (Fig. 2) do not show a distinguishable difference in binding between the humanized clones and the chimeric parent Fab, we believe that the difference in the measured k_{off} between the clones and parent reasonably approximates the difference in affinity. The difference in k_{off} measured for humanized BR96 Fabs, therefore, represents less than a 2-fold loss in binding affinity relative to the BR96 M1 molecule.

TABLE II

Dissociation rate constants of humanized BR96 M1 clones

Dissociation rate constants were measured by surface plasmon resonance (BIA-core™; Biosensor, Piscataway, NJ) by using sLe^y conjugated to human serum albumin immobilized onto a derivatized metal sensor chip. Fabs isolated in periplasmic fractions from *E. coli* infected with the parent control BR96 M1 and the humanized BR96 phage were analyzed as described in Yelton *et al.* (34).

Fab	k_{off}	Difference relative to BR96 M1
	s^{-1}	*-fold*
BR96 M1	0.041	1.0
1-29	0.069	1.7
1-76	0.064	1.6
1-88	0.069	1.7
2-31	0.065	1.6
2-40	0.068	1.7

Sequencing of Humanized Fabs—Table III lists the amino acids found at each of the library positions in the five humanized clones. In the light chain, all five clones had the murine residue Tyr at 36 and the human residue Gln at 37. The murine residue Leu at position 37 does not affect binding activity, since another clone with similar activity to the five clones characterized in this study had Leu at that position (data not shown). Furthermore, introduction of Leu at 37 into a humanized clone by site-directed mutagenesis showed that the human residue Gln could be replaced without a loss of activity (data not shown). Sequencing identified both murine and human residues at light chain positions 11, 43, and 104 in different humanized clones. Clone 1–76 had Pro at light chain position 11, which is neither the murine nor human alternative, and it presumably resulted from a PCR error. Consequently, we are evaluating alternative polymerases such as *Pfu* and Vent™ to eliminate errors introduced by *Taq* polymerase. At the heavy chain library positions, all clones selected had the human amino acid residues, Ala at position 24 and Ser at 49.

The clone chosen for further study, 2-40, had human template residues at five of the seven library positions. The murine residues at positions 36 and 43 in the light chain are present in the consensus sequence for approximately 50 of the most homologous human heavy chain sequences to BR96 (Fig. 1), diminishing our concern about potential immunogenicity. Furthermore, all framework residues in humanized BR96 are represented in the human template or subgroup consensus for both heavy and light chains except for position 2 in the light chain, a structural determinant residue we chose not to mutate in this study. We have subsequently compared the human Ile residue at position 2 to the murine Val and have shown that binding was equivalent in both cases (data not shown).

Site-directed Mutagenesis of Humanized BR96—A recent report described the humanization of B3 (20), another mAb that recognizes the Lewis Y class of tumor antigens (26). B3 and BR96 have highly homologous light chain and heavy chain V region sequences, 96 and 85%, respectively (27) (Fig. 3).

TABLE III

Amino acid sequence of library positions in humanized BR96 M1 clones

The heavy chain and light chain V regions of five humanized BR96 M1 clones were sequenced as described under "Experimental Procedures." The amino acids at the combinatorial library positions in the murine BR96 V region, in the human template sequences, and in the five humanized clones are presented.

Construct	Position[a]						
	H24	H49	L11	L36	L37	L43	L104
mBR96 M1	Thr	Ala	Leu	Tyr	Leu	Ser	Leu
Human template	Ala	Ser	Ser	Leu	Gln	Pro	Val
1–29	Ala	Ser	Ser	Tyr	Gln	Pro	Val
1–76	Ala	Ser	Pro[b]	Tyr	Gln	Pro	Val
1–88	Ala	Ser	Leu	Tyr	Gln	Ser	Leu
2–31	Ala	Ser	Leu	Tyr	Gln	Ser	Val
2–40	Ala	Ser	Ser	Tyr	Gln	Ser	Leu

[a] H designates heavy chain, and L designates light chain.

[b] Proline was not included as a library residue and resulted from a PCR error.

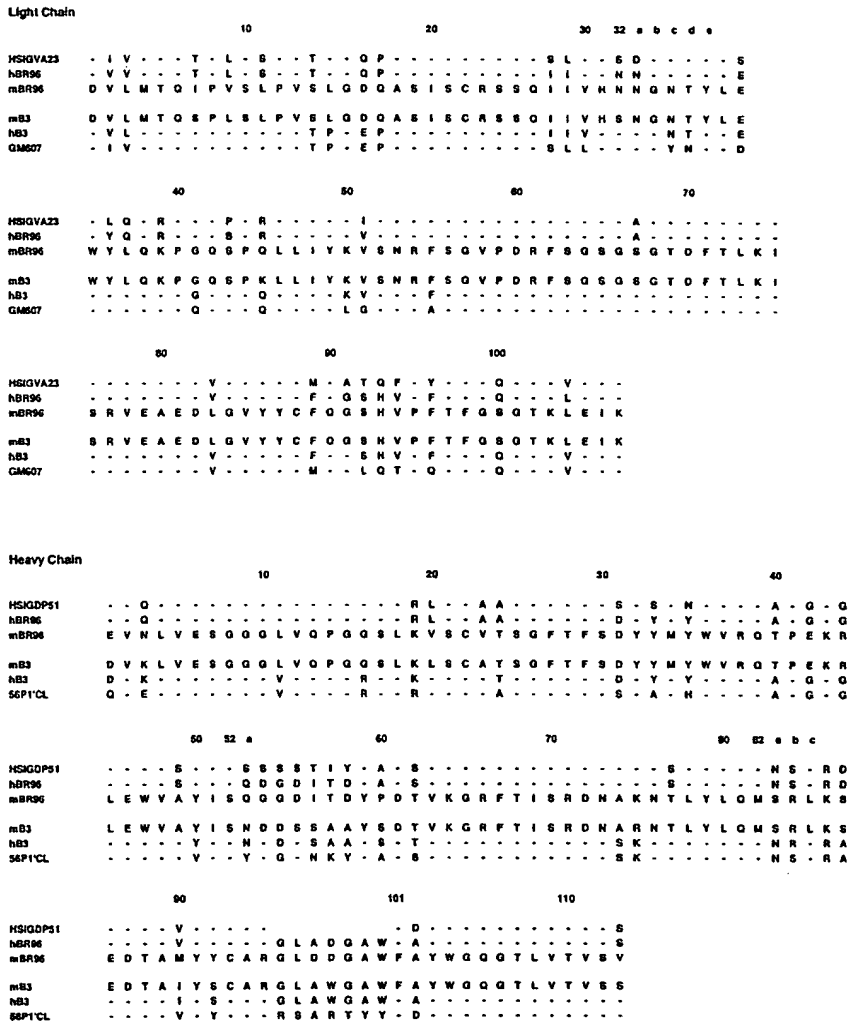

FIG. 3. **Alignment of murine and humanized BR96 sequences with murine and humanized B3 sequences and human templates for each.** *A*, alignment of mBR96 light chain, hBR96 light chain, and human template HSIGVA23 with murine B3 (*mB3*) and humanized B3 (*hB3*) light chains, and human template, GM607 (20). *B*, alignment of mBR96 and hBR96 heavy chains and human template HSIGDP51 with mB3 and hB3 heavy chains and human template, 56P1'CL (20). Amino acids that are identical to the murine amino acid are designated by a *dash*.

We compared the residues humanized in each antibody and particularly noted the positions in B3 identified as important to antigen binding. The humanized B3(Fv)-PE38 immunotoxin, as originally designed, showed a 20-fold loss in activity, but two site-specific changes resulted in substantial recovery of ligand binding and cytotoxicity (20). Mutation of Leu^{104} (murine) to Val^{104} (human) in the B3 light chain restored binding activity 8-fold. The authors suggested that humanization of light chain residues 15 and 18, both to Pro, required that 104 also be humanized. For the humanization of BR96, the combinatorial library included light chain position 104 with the same amino acid alternatives as for B3. We found that substitution of either amino acid at that position in humanized BR96 M1 did not affect antigen binding. BR96 has Leu at position 15 not Pro, which may account for greater flexibility to substitute residues at 104.

An additional 2-fold recovery in binding of humanized B3 occurred by replacing human residue Ser with the murine residue Arg at position 82b in the heavy chain. In humanized BR96 we had also substituted the human Ser for the murine Arg so we questioned whether changing the residue at 82b in BR96 might affect its binding activity. We changed the human residue Ser back to the murine amino acid, Arg, at 82b by site-directed mutagenesis. The clone used for this experiment was humanized BR96 M3, a derivative of clone 2-40 with an additional affinity mutation, Gly to Asp at 53 in the heavy chain (34). The return to the murine residue did not alter binding to tumor (Fig. 4). The k_{off} measured by SPR was also unchanged (data not shown).

Residue 19 of humanized B3 heavy chain fit the criteria that Benhar *et al.* (20) had established for humanization of B3. However, based upon their unpublished observations they suggested that mutations introduced at residue 19 resulted in substantial activity loss, and despite the conservative nature of the change (Lys to Arg), they retained the murine residue. We had chosen to humanize position 19 in BR96. To test whether this position affected antigen binding of humanized BR96, we changed the Arg back to the murine residue Lys. As shown in Fig. 4, changing the residue had no effect on binding activity. The k_{off} of this molecule measured by SPR was similar to that of humanized BR96 M3 and the 82b site-directed mutant (data not shown). Thus, none of the positions that had or were suggested to have an impact on the humanization of B3 had a significant effect on humanized BR96 binding, despite the great sequence homology and similar antigen specificity of the two antibodies.

DISCUSSION

Many "humanized" forms of a monoclonal antibody are possible. A compromise between humanizing a murine framework residue to reduce its potential immunogenicity and retaining a residue critical to antigen binding and specificity is often nec-

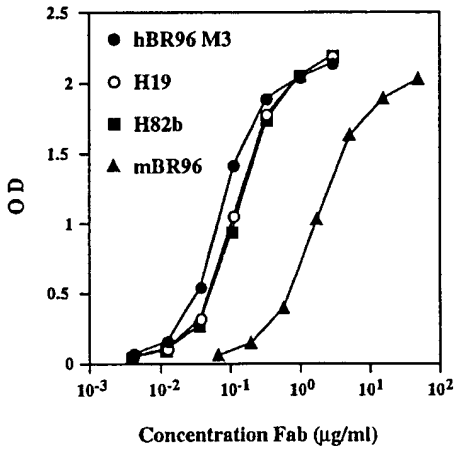

FIG. 4. **Binding of humanized BR96 M3 site-directed mutants to tumor cell membranes.** Murine residues at positions 19 and 82b in the heavy chain were introduced into humanized BR96 M3 by site-directed mutagenesis. Binding of the Fab fragments in bacterial periplasm fractions to H3396 tumor membranes was compared by ELISA. mBR96 is proteolytically derived Fab prepared as described in Fig. 2.

essary. We have presented a combinatorial library strategy to examine murine and human alternative residues at positions potentially important for retaining antigen binding. Simultaneous evaluation of antigen binding of Fabs representing all combinations of selected residues eliminates repeated rounds of design and analysis frequently required to identify the "most" human sequence that maintains the best binding.

Selection of the human frameworks to use as templates for humanization of foreign V regions defines subsequent decisions regarding which residues to humanize. Choosing homologous templates from antibodies with known crystal structure, from germline, non-germline, or consensus sequences derived from available data bases are options (for a review, see Routledge *et al.* (43)). As discussed in more detail below, the choice of templates for the light chains of BR96 and B3, germline template for BR96, and a non-germline sequence for B3, dictated that different residues be humanized in the two antibodies. Even though we chose germline sequences to eliminate potential immunogenicity of somatic mutations, no particular template selection method has proven superior to another regarding immunogenicity. Too few humanized antibodies have been administered clinically to provide sufficient data about the immunogenicity of humanized mAbs.

A patient's immune response to solvent exposed residues of a mAb results in more rapid clearance of the mAb upon subsequent administrations (43). Therefore, we preferred to humanize all the solvent exposed residues to minimize immunogenicity. Although we wished to humanize as many residues as possible, we were hesitant to change residues at the light and heavy chain V region interface or adjacent to CDR loops, since they may affect antigen binding. Many of these amino acids are buried or "packing" residues (33) and are important to antigen binding (11, 22). Positions selected for the BR96 library included the buried positions for which the murine and human alternative amino acids represented nonconservative changes.

A library strategy is advantageous because it can incorporate any position for which the choice of human or murine amino acid is ambiguous. General structural information compiled from crystallized antibodies, *e.g.* CDR loop conformation (13), identification of positions as high, medium, or low risk for humanization (44), degree of solvent exposure (33), or antibody-specific modeling information can be useful when deciding to retain the murine residue, change it to human, or incorporate it as a library position. Furthermore, rodent and human V regions share homology in CDR sequences, and future attempts at humanization could include selected CDR residues as well as framework residues.

Humanization of buried residues did not adversely affect binding of BR96 to antigen. Several humanized BR96 M1 Fabs demonstrated binding activity comparable to the murine V region parent. In five clones sequenced, we found only the human residue at three positions, both human and murine residues at three positions, and only the murine residue at one position. The murine residue Tyr predominated at position 36 in the light chain and it also dominates in the human sequences homologous to BR96. This position is involved in the interface between the V regions of the heavy and light chains, and so it may not be permissive to change. The predominance of human residues at three positions was unexpected, but because we sequenced only five clones, we do not think that the data statistically prove an advantage of human over murine residues at these positions. While the binding activity of all selected clones was similar to mBR96 Fab, the expression of the humanized clones was higher in the bacteria. The humanized Fabs expressed in the bacterial periplasm all yielded 4–8-fold more Fab than typically found for the murine V region. The plaque lift assay used initially to select clones from the library is sensitive to expression level. The human residues that predominated in some clones could have affected their expression in bacteria, but we cannot distinguish whether library positions or the other humanized residues not included in the library caused this improvement. Application of a combinatorial strategy might also be useful solely to maximize protein expression, often an important issue in bacterial expression of mammalian proteins.

The different approaches taken to humanize BR96 and B3 illustrate how template choice and form of an antibody can influence the outcome of the process. We chose germline sequences for humanization, whereas Benhar *et al.* (20) selected the most highly homologous templates without the same constraint from their data base search. The BR96 heavy chain framework has sufficient dissimilarity to B3 that the 46 human sequences most homologous to BR96 did not include the template used for B3. The light chains, on the other hand, have only three amino acid differences in the frameworks, and homologous sequences identified in our data base search did include the template for B3, GM607. If we had chosen GM607 as template, our choice of buried residues for the library would have been reduced to one, position 104. The different choice of templates not only determined library composition, but also led us to mutate a somewhat different set of solvent-exposed residues. Ultimately, the two humanized mAbs had greater divergence in sequence than the original murine antibodies.

Humanization of B3(Fv)-PE38 as originally designed resulted in a molecule with a 20-fold loss in binding activity. By iterative design and analysis they identified the framework positions critical to recovery of binding activity. Despite the sequence similarity of B3 and BR96, humanization of positions or preservation of particular murine residues in BR96 was not critical to its activity. Because B3(Fv)-PE38 immunotoxin is a single-chain fusion protein isolated from inclusion bodies in *E. coli* and requires refolding, alterations to the amino acid sequence of the Fv could affect the refolding process. These amino acid substitutions might not affect BR96 similarly, since it is expressed as a soluble functional Fab.

Humanization is an empirical process and many modifications of xenogeneic antibodies are conceivable that might fit a definition of humanized. In this report many humanized BR96

Fabs with varied humanized sequences that bound comparably to tumor antigen and without significant loss in affinity were rapidly identified. Combinatorial library strategy offers a flexible approach to the humanization of antibodies and to other protein engineering projects.

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